MODULATION OF CARDIAC MACROPHAGES WITH INTERLEUKIN-4 AS A STRATAGEY OF INFARCT HEALING

A Thesis
Presented to
The Academic Faculty

by

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In Partial Fulfillment
of the Requirements for the Degree
Bachelors of Science in the
School of Biomedical Engineering

Georgia Institute of Technology
May 2015

MODULATION OF CARDIAC MACROPHAGES WITH INTERLEUKIN-4 AS A STRATAGEY OF INFARCT HEALING

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Date Approved: December 7th, 2015

ACKNOWLEDGEMENTS

I would like to express my deep gratitude to my parents for their continuing support and encouragement. I would like to thank my mentor Inthu, for sticking with me for these three years and providing me endless opportunities to enhance my scientific interests. I would like to thank principle investigator Dr. Michael Davis for entrusting me and believing in my capabilities.

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LIST OF SYMBOLS AND ABBREVIATIONS

MI	Myocardial Infaction
M1	Pro-inflammatory Macrophage
M2	Wound Healing Macrophage
LPS	Lipopolysaccharide
IFNy	Interferon Gamma
IL4	Interleukin 4
NO	Nitric Oxide
Arg1	Arginase 1
MRC1	Mannose Receptor C Type 1
PEG-MAL	Polyethylene Glycol Maleimide
MSC	Mesenchymal Stem Cells
CM	Conditioned Media
CPC	Cardiac Progenitor Cells

SUMMARY

Cardiovascular disease is the leading cause of death in the United States accounting for approximately 1 in every 3 deaths. Many efforts have been made to reduce the mortality rate after a MI by analyzing and possibly manipulating the immune system response following an MI. Immediately after an MI has occurred, pro-inflammatory macrophages (M1) are recruited at the affected site. M1 are responsible for clearing dead cells and debris. Following the M1 response, wound healing (M2) macrophages, responsible for angiogenesis and cell regeneration, are recruited at the site. Although the M1 subset is required for proper wound healing, prolonged activation of this macrophage subset attributes to cell fibrosis and tissue scaring. This study initially aims in modulation of macrophages to a M2 phenotype utilizing IL-4 after an MI occurs. The secondary aim is delivery of IL-4 to the heart site utilizing a PEG-MAL hydrogel. This study could prove that delivery of IL-4 to the heart could suppress inflammatory signals after an MI and lead to a healing response. *In vitro* studies in RAW and bone marrow macrophages indicated IL-4 leads to an increase in the MRC1 and Arginase 1, M2 markers, and macrophages stimulated with lipopolysaccharide (LPS) and interferon gamma increased M1 markers nitrite and tumor necrosis factor alpha. The addition of both M1 and M2 stimuli simultaneously increased Arginase 1 and nitrite production significantly. The IL-4 treated media from the *In vitro* macrophages increased cardiac progenitor cell migration by 70% relative to the control. This shows that IL-4 differentiated macrophages can communicate with other cells crucial to healing. Knowledge of the effects of IL-4 after a heart attack could greatly improve cardiac cell regeneration and lead to better patient health.

CHAPTER 1

INTRODUCTION

Background

Cardiovascular disease is the leading cause of death in the United States accounting for approximately 1 in every 3 deaths. Approximately 83.6 million American adults have one or more types of cardiovascular disease in which 7.6 million people are subject to myocardial infarction (MI), which is the most common cause of heart failure.[1] An MI is caused by a blockage in the coronary artery that delivers blood to the left ventricle. [1] After a MI occurs, there is a substantial loss of cardiomyocytes. In most cases, a non-contractile scar formation in the myocardium occurs and leads to impaired ventricular function. A key factor in this processes is the inflammatory response to the injury.[2] Most of the efforts to repair the heart after a MI have focused on stem cell, gene or growth factor delivery to the injured heart, but yet to be clinically successful.[3] However, Macrophages, central to this inflammatory process have been largely overlooked as therapeutic targets. Recent studies have shown that immediately after an MI has occurred, pro-inflammatory macrophages (M1) are recruited at the affected site. M1 macrophages are responsible for clearing dead cells and debris. Following the M1 response, anti-inflammatory (M2) macrophages, responsible for wound healing and tissue repair, are recruited at the site. [4] The pro-inflammatory immune response lasts around four days, the shift towards the anti-inflammatory immune response takes place between day 4 and 7. [4]Although the M1 subset is required initially, prolonged activation of this macrophage subset attributes to adverse cardiac remodeling characterized by fibrosis and non-contractile scar tissue formation. [5,6] Therefore, optimal healing response after MI requires a balancing act of the biphasic macrophage response. Activation of M2 macrophages earlier during the course of inflammation following MI via external

intervention could trigger repair mechanisms and release of complementary factors that can bias cardiac cell towards a regenerative outcome. Thus, modulating the immune response could hold tremendous potential as a therapeutic strategy for chronic MI. This research aims to modulate the cardiac macrophage phenotype in the heart by delivering the M2-polarizing cytokine IL-4 using a hydrogel delivery system in a rat model of experimental MI. This treatment is expected to favorably shift the macrophage subsets in the infarcted heart from pro-inflammatory to a reparative phenotype and create a pro-regenerative environment to better heal the heart.

Macrophage Activation

The M1 and M2 phenotypic characterizations are actually extremes of a spectrum of phenotypes that vary in functionality: host defense, wound healing, and immune regulation.[7] *In vitro* the stimuli for macrophage activation it is well characterized. M1 activation is induced by lipopolysaccharide (LPS) and Th1-related cytokines, such as interferon gamma (IFNy), where as M2 activation is induced by glucocorticoids or Th2-related cytokines.[8] The M2 subset is further divided into M2a, M2b, and M2c. M2a, characterized by the wound-healing phenotype, is activated by interleukin 4 (IL-4) and interleukin 13. M2b and M2c are characterized with a regulatory phenotype. M2b activation is achieved through immune complex with toll-like receptor ligand and M2c activation is induced by IL-10.[7,8] Although *In vitro* macrophage activation is well characterized, it is unable to mimic the complex environment observed in *In vivo* activation. When macrophages are polarized to M1 or M2 (M2a) they express specific characteristics that can be used as markers for identification of these phenotypes. M1 macrophages upregulate nitric oxide (NO) production and pro-inflammatory cytokines such as IL-12 and TNF-alpha while M2 macrophages increase expression of Arginase 1 (Arg1) and macrophage mannose receptor C type 1 (MRC-1).[7,8]

Hydrogel Delivery System

Since the heart tissue is a highly vascularized environment, direct administration of a small protein such as IL-4 will be highly inefficient. Previous studies in our laboratory have shown promising use of a polyethylene glycol-Maleimide (PEG-MAD) based protease sensitive hydrogel to deliver therapeutics to infarct tissue in experimental MI models.[9] Since injectable hydrogel elicits no inflammatory response, it was used as a model for our PEG-MAL hydrogel. The PEG-MAL hydrogel is composed of a 4-arm PEG macromere end functionalized with maleimide groups that allow for functionalization with any cystine containing peptides and a cell adhesion molecule (RGC) (figure 1). The main component of this hydrogel is the protease sensitive peptide cross-linker (VPM). As the dominant M1 macrophage population in the post-MI heart upregulates proteases in the environment, hydrogel degrades over time thereby facilitating the sustained release of IL-4.

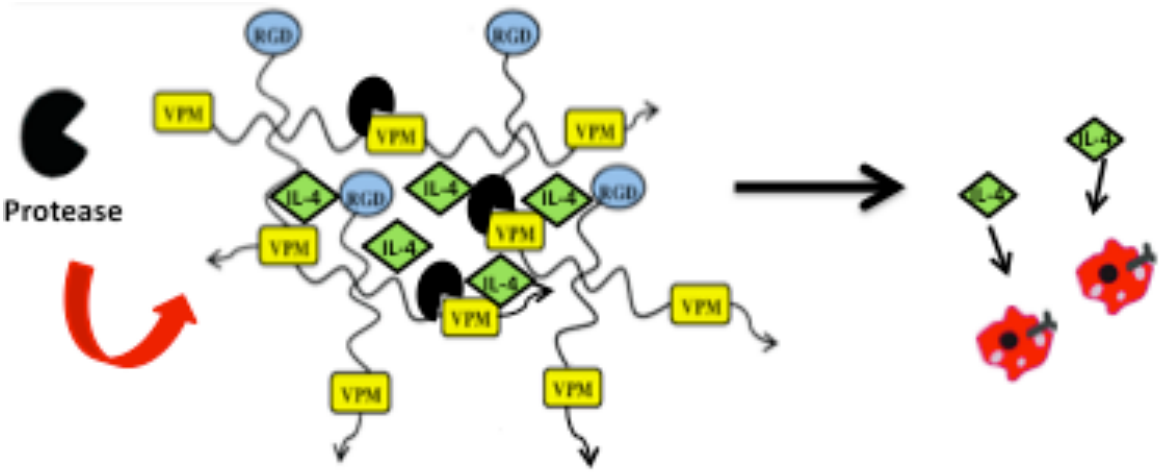

Figure1. PEG-MAL Hydrogel Delivery Mechanism.[9] Proteases present in the environment cleave at VPM releasing IL-4 at the injury site.

CHAPTER 2

LITERATURE REVIEW

The regenerative properties resulting from the switch in the activation types of macrophages have been analyzed in several disease and regeneration models[10,11]. For instance, similar to the heart following MI, in skeletal muscle repair, initially, the injured muscle only recruited the pro-inflammatory phenotype; however a shift occurred in which the muscle later recruited anti-inflammatory activated macrophages.[12] This phenotypic switch stimulated myogenesis and fiber growth. Further, in a study induced peripheral nerve repair using M1 or M2 activated macrophages.[10]

Similarly, efforts to manipulate macrophages to bring about regenerative effects have been attempted on several different models. In a peripheral never regeneration model, when conduits containing IFN-y (to shift macrophages to M1 phenotype) or IL-4 (to shift macrophages to M2 phenotype) was implanted in the nerve gap injury, it was demonstrated that IL-4 containing conduits greatly increased the proliferation of Schwann cells and axons through the conduits.[10] This strategy is very similar to the one we are implementing for cardiac repair following MI and further provides validation that it could be a viable strategy.

Further, in an MI model, phosphatidylerine (PS) presenting liposomes mimicing apoptotic cells were used [13] to switch pro-inflammatory macrophages to anti-inflammatory macrophages. M2 activation has been observed with intravenous injections of phosphatidylerine (PS) – presenting liposomes that adhere to the macrophage and mimic a “death” signal. This signal recruits anti-inflammatory macrophages to the heart following a MI. PS-presenting liposomes affectively increased of the CD206 marker for M2, and decreased the M1 marker CD86. In addition, the M2 phenotype was expressed at least a day earlier than the time frame usually observed (day 4-7) following MI than compared to the control groups that received MI but not the PS-liposome treatment.

However, it is unknown whether the PS-presenting liposomes mimicking apoptotic cells could have adverse effects systemically. Our approach utilizes a hydrogel delivery system to directly target the activated macrophages in the heart.

One of the aspects of our study is to assess the paracrine effect of the activated macrophages, specifically stem cells. This is done by collecting the conditioned media from activated macrophages and analyzing and testing the effect of this media on other cell types of interest. Several studies in the literature illustrate similar concepts. In one study, neuron survival and neurite regeneration induced by myelin-activated macrophages were assessed. After four days, *in vitro* data showed the myelin-activated peritoneal macrophage conditioned media had about 56% survival of the neurons compared to the control of just macrophage condition media with 40% survival.[11] The mechanism for neural regeneration by this myelin-activated macrophage is unknown. It is possible that the myelin was ingested by the macrophages and are causing phenotypic changes, or changes in the secretome (proteins, microRNA etc) of these macrophages.

Our approach utilizes cytokine IL-4, a molecule traditionally known to bring about M2 phenotype. Several studies in literature have utilized various other approaches to activate macrophages towards M2 phenotype. In one study, co-culturing mesenchymal stem cells (MSC) with peripheral blood monocytes caused M2 phenotype in these monocytes, and this was attributed to the regenerative properties of MSCs. In vitro data showed an increase in the M2 marker CD206 and anti-inflammatory TH2-related cytokine IL-10[14]. However, the MSC co-cultured macrophages also retained high levels of inflammatory cytokines. This new macrophage subset now has both properties of the M1 and M2 phenotype and therefore it is unclear whether this approach is effective in reducing inflammation. Another study utilized SEW2871, a macrophage recruiting agent, in conjunction with platelet rich plasma (PRP) implanted in a hydrogel to analyze their effects on bone remodeling.[15]

Currently, research with regard to macrophage activation utilizing hydrogels applied to the cardiac region is not prevalent. Our aim is to encapsulate IL-4, a known macrophage modulator, in a hydrogel and deliver it to the heart in order to shift the pro-inflammatory phenotype to the anti-inflammatory phenotype in the heart earlier than the pre-determined time. We believe that this strategy could reduce the overwhelming pro-inflammatory response and enhance anti-inflammatory macrophage response in the injury site in order to enhance cardiac repair.

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CHAPTER 3

MATERIALS AND METHODS

Firstly, we aim to validate our treatments *In* vitro. Our goal is to determine if macrophages can be activated through IL-4 and IFNy plus LPS. Additionally, we want to characterize wound-healing properties that are associated with our treatment. Finally, we want to test our approach *In vivo* and assess the functionality.

RAW Macrophage Modulation

RAW macrophages are a widely accepted cell line for *In vitro* experiments due to their long life span. In a 6-well tissue culture plate, about a million macrophages were plated in each well. Following adherence of the cells, media was changed to serum free media to quiecse the cells for 4 hours following which the cells were either treated with 10ng/mL of IL-4 for M2 activation or 10ng/mL of IFN-y and 0.1ug/mL of LPS for M1 activation while one treatment group just contained serum free media as untreated control. After 24 hours, the conditioned media (CM) from each well was collected to determine NO production via Greiss assay. The cells were extracted using Trizol reagent for RNA isolation and gene expression analysis conducted via qPCR. The characteristic markers of M1 and M2 phenotypes were analyzed in samples from all treatment groups and compared against the untreated control group. To determine the gene expression levels, qPCR was used with gene-specific primers. The M1 marker TNF-a and M2 markers Arg 1 and Mrc1 as well as the house-keeping gene Hprt1 to normalize gene expression were analyzed. The ΔΔCT method was used to quantify fold changes in gene expression normalized to Hprt1 mouse house-keeping gene.

Bone Marrow Macrophage Modulation

Bone marrow derived macrophages more accurately represent the internal environment due to the fact that they are a primary cell line. Therefore, our treatments were applied to these primary cells. Bone marrow was harvested in the femur and tibia of a rat. The cells were plated for seven days on a petri dish in 20% FBS RPMI media. The

cells were then allowed to adhere to a T-75 cell culture plate for a day. In a 6 well tissue culture plate, approximately 1 million cells/mL were treated with 10ng/mL of IL-4 (for M2 activation) or 1ng/mL of IFN-y and 0.1ug/mL of LPS (for M1 activation) in serum free media. After 24 and 48 hours, the conditioned media from each well was collected to determine NO production via Greiss assay and the cells were scraped in PBS to be used for an Arginase assay.

Cardiac Progenitor Cell (CPC) Migration

. Cardiac progenitor cells have been increasingly identified as a new therapy for cardiac repair post MI. Increased recruitment of CPCs can lead to an increased wound-healing response. CPCs were fluorescently labeled with CMRA in serum free Ham's media. Using a modified Boyden chamber assay in a 24-well plate, 100,000 CPCs in 100uL were placed in the top chamber while 300uL of treated macrophage CM was placed in the bottom. The plate was incubated in 37 degrees Celsius overnight and read with a fluorescent plate reader.

Quantification of Myocardial Fibrosis

After exposure to a MI, through occlusion to the coronary artery, evaluating the ratio of fibrotic to healthy heart tissue will assess the effects of our treatment *In vivo*. The animal was sacrificed and the heart was embedded in paraffin. Sections 7um thick were mounted onto slides. After submersion in Histoclear for 15 min, the section was rehydrated in a series of ethanol dilutions (100%,90%,70%) and placed in picoeirius red for 1 hour. The section was allowed to dry for 15 min, before the cover slip was mounted.

CHAPTER 4

RESULTS

RAW Macrophage Modulation

In a RAW macrophage cell line, IL-4 treated cells exhibited a significant increase MRC-1 and Arg1 gene expression, which indicates activation towards an M2 functional phenotype, while IFNγ plus LPS treated cells show increased expression of TNF-a and high levels of NO, characteristics of M1 macrophages (n: 1-3, p <0.05) (figure 2). These results confirm the plasticity of macrophages and our ability to modulate their phenotype using external stimuli.

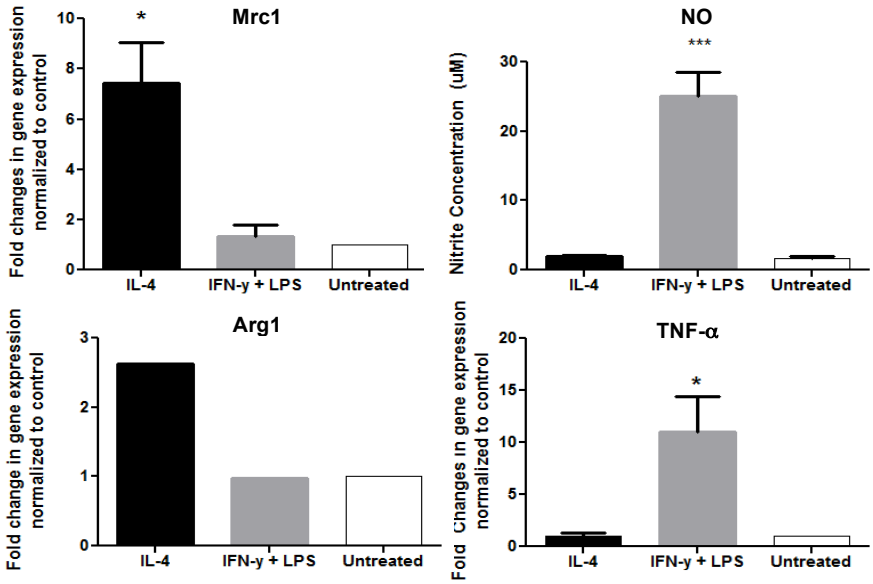

Figure 2. Characterization of macrophage activation markers. All gene expression was normalized to housekeeping gene hprt1 (n=1-3, *p<.05, ***p<.0001).

Bone Marrow Macrophage Modulation

Macrophages treated for 48 hours with IFNγ with LPS (M1) and IL-4 (M2) showed a significant increase in nitric oxide (figure 3) and Arginase 1 (figure 4) respectively, which coincides with the RAW macrophage data. However when macrophages are treated for 24 hours with IFNγ plus LPS and then treated for 24 hours with IL-4, the

conditioned media has high concentrations of nitric oxide. When macrophages are simultaneously treated with IFNy plus LPS and IL-4 for 48 hours, cells express high concentrations of Arginase 1. This suggests that once macrophages are polarized down an M1 lineage it is more difficult to shift the macrophages to an M2 phenotype. The simultaneous activation shows that it is more advantageous to have IL-4 present in the environment as soon as macrophages arrive to the injury site to achieve a greater potential for M2 polarization.

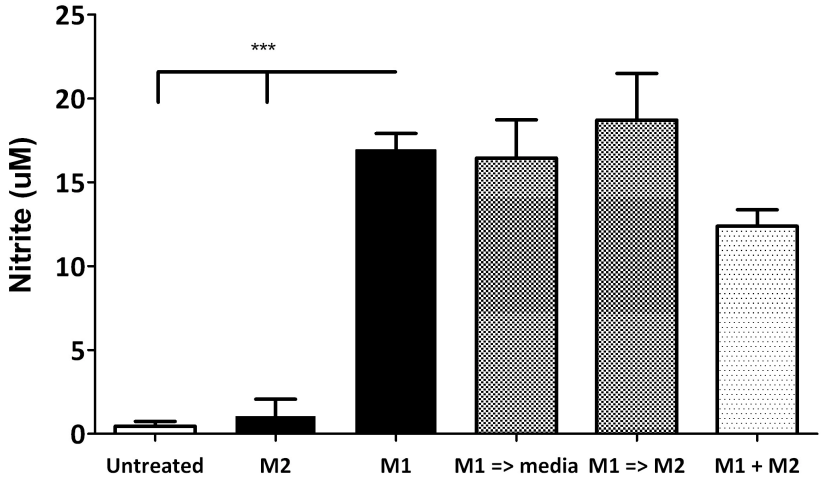

Figure 3. Nitric oxide concentration from bone marrow macrophage conditioned media. All media was used after 48 hours exposure to treatment. (n=5, *p<.05, ***p<.0001).

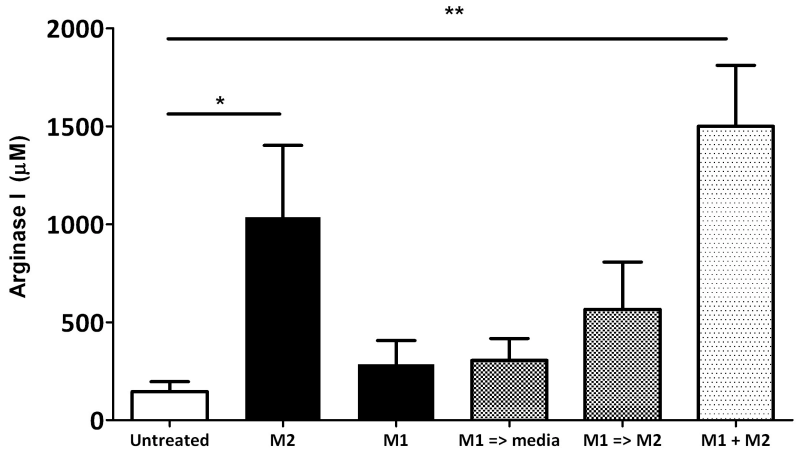

Figure 4. Arginase 1 concentration from bone marrow macrophage conditioned media. All media was used after 48 hours exposure to treatment. (n=5, *p<.05, **p<.01).

CPC Migration

The percentage of CPC migration increased 70% with IL-4 CM relative to the control. However we also wanted to assess the migratory capabilities of just the treatments alone in the media. This concluded little variation in percent migration due to the treatment alone, which confirms that the CPC migration from IL-4 CM is not just due to the IL-4 in the media, but due to the factors released by M2 macrophage subset. Therefore, the factors that the M2 macrophage subset secrete into the environment are drawing CPCs to the injury site to aid in tissue repair.

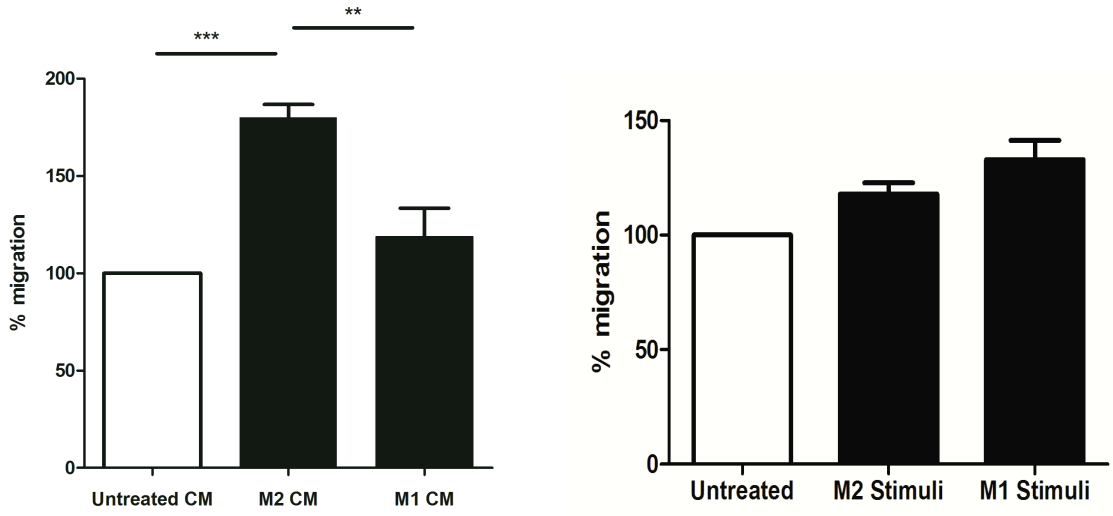

Figure 5. Percentage of CPC migration. All conditioned media was used from 48 hours of treatment exposure (n=1-3, *p<.05, ***p<.0001).

Quantification of Myocardial Fibrosis

The quantitative data was not significant however, qualitative analysis potentially shows a decrease in fibrotic tissue (red) and increase in healthy tissue (yellow) from the MI heart to the IL-4 hydrogel treated MI heart 21 days after the MI occurred (figure 6). This suggests that the IL-4 hydrogel treatment could have long-term regenerative properties, which is beneficial for treating chronic MI.

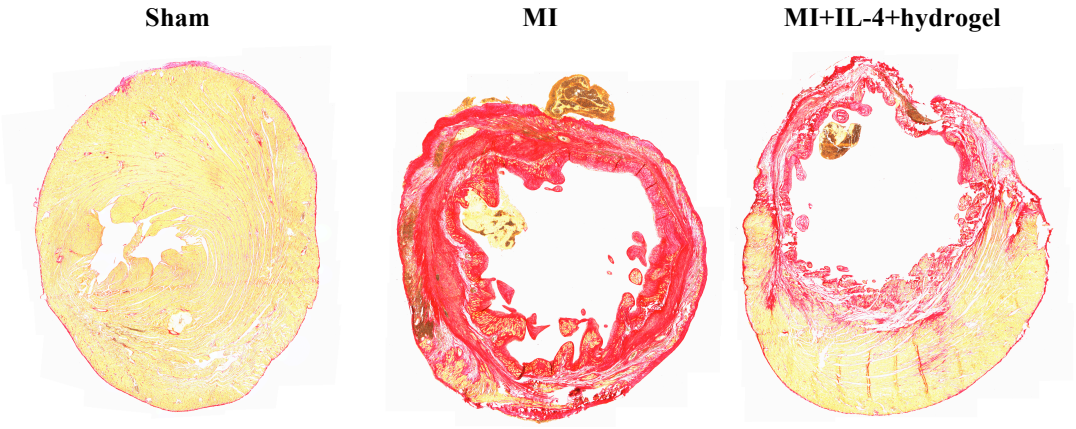

Figure 6. Myocardial fibrosis day 21. All sections were captured under the same exposure time

CHAPTER 4

DISSCUSION

In vitro data proved that when RAW and bone marrow derived macrophages are treated with either IFNY plus LPS or IL-4 macrophages will polarize towards an M1 or M2 subset respectively. We saw an increase in the expression of MRC-1 and Arg1 with the M2 polarized macrophages and an increase in TNF-a expression and NO production in M1 polarized macrophages. Additionally, we saw the effects of staggered versus simultaneous activation. Simultaneous activation is more beneficial for polarizing macrophages to the M2 phenotype. Once macrophages are polarized to the M1 phenotype, they have less potential to polarize to the M2 phenotype. Therefore, it's crucial that IL-4 is present in the environment once macrophages arrive to the injury site. In order to determine the wound healing effects of IL-4 treated macrophages in vitro, we aim to evaluate cardiac progenitor cell (CPC) recruitment due to the CPC's effect on the viability of cardiac cells. Although the fibrosis staining of treated heart tissue was not significant, a gene and/or protein expression analysis could determine the healing potential of IL-4 treated macrophages post MI. If IL-4 posses healing potential in vitro, application of IL-4 after a myocardial infarction could increase the patient quality of life and decrease further complications.

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